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13. ABSTRACT (Maximum 200 words)			

Our laboratory, during an earlier AFOSR granting period, was the first to isolate and clone a full-length cDNA for this enzyme. We also showed that this cDNA, in an appropriate vector, can be expressed in eukaryotic cells above endogenous levels. Accordingly, our laboratory is capable of performing direct experiments utilizing recombinant DNA techniques, to test for the role of this enzyme in DNA repair and recovery from toxic agents during the renewal period. For example, we propose to construct expression vectors containing alterations in the active site and the DNA binding domain of PADPRP and to eventually stably integrate these into eukaryotic cells such that expression of these "analog" PADPRPs will be expressed. Through the use of several of these mutants that we have already expressed in *E. coli* during the past granting period, the modulation of PADPRP structure should allow us to learn considerably more about the mechanism and role of this enzyme in cells exposed to stressful environments.

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ANNUAL TECHNICAL REPORT

THE KEY INVOLVEMENT OF POLY(ADP-RIBOSYLATION) IN DEFENSE
AGAINST TOXIC AGENTS: MOLECULAR BIOLOGY STUDIES

APRIL 29, 1993

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A. Statement of Work

It is commonly realized that NAD is synthesized within the eukaryotic nucleus and represents a major metabolite the organelle. approximately 95% of the nuclear NAD is utilized by PADPRP a chromatin-associated enzyme which poly ADP-ribosylations a selected pool of those chromosomal proteins (histones, topoisomerase I and II, large T antigen, and perhaps c-fos and c-myc) that perform legitimate functions adjacent to DNA strand breaks PADPRP requires DNA for activity; a major clue toward ascertaining the function of the enzyme has been the observation described by Benjamin and Gill that the catalytic activity of PADPRP is directly coordinated to the numbers of strand breaks in DNA. Thus, relevant to the objectives of the AFOSR project is the fact that PADPRP causes the poly ADP-ribosylation of nucleosomal proteins in response to a variety of DNA-damaging agents including, ionizing irradiations. Our laboratory in 1975, and that of Shall, were the first to show activation of PADPRP by DNA damaging agents. It was also shown that an accompanying drop in cellular NAD may be involved in the cytotoxic effects of alkylating agents, which stimulated the rationale of current Air Force Renewal project. For example, number of fuel components contain polyacromatic hydrocarbons, naphthalenes, etc. many of which, upon activation, can cause DNA strand breaks, hence, their cytotoxic effects would be expected to be modulated by poly ADP-ribosylation. Equally many pesticides also fit into this category. It is therefore of some interest to this project that work performed nearly 14 years by Preston and Candido showed that a large number of organophosphorus and methylcarbonate insecticides, in very low doses, cause dramatic lowering of NAD (activation of nuclear ADP-ribosylation?) in chick embryos which correlated very well with the teratogenic effects of these compounds.

During the previous granting period of this project, we characterized the various levels of interaction of PADPRP and with respect to genetic and also chromatin structure. The past work has included cloning and sequencing the relevant genes for PADPRP from human and mouse and work on the elucidation of the regulation of expression of PADPRP mRNA. Additionally, feasibility studies, have been performed during the initial period of the current granting year on expression of "analog PADPRP proteins in cells."

B. Status of Progress Towards Research Objectives

Progress During Year 1 on Aim I: (Specific Aim I: EXPRESSION OF "ANALOG" PADPRPs IN MAMMALIAN CELLS: ROLE AND MECHANISM OF PADPRP IN DNA REPAIR)

In Aim I we initially proposed in the Renewal Application (1992) to question whether mutations generated in selected functional domains of PADPRP (initially exploiting bacterial mutagenesis) would be useful with eukaryotic express vectors to ascertain the role of this enzyme in mammalian cells in recovery from cytotoxic agent. Would the expression and thus nuclear localization of an "analog" PADPRP lacking active sites of the enzyme in eukaryotic cells compete with endogenous PADPRP during DNA repair and provide a new method to ascribe the role of this enzyme in DNA strand break recovery? Can the DNA binding domain of PADPRP be "swapped" with the sequence-specific glucocorticoid receptor (GR) DNA binding and be expressed in *E. coli*?

Initial progress on the background for this aspect of the applications was provided by a recent publication from our laboratory on the expression of various analogs of PADPRP in *E. coli* (Cherney, *et al.* Expression and mutagenesis of human poly (ADP-ribose) polymerase as a ubiquitin fusion protein from *Escherichia coli*. *Biochemistry* 30, pp 10420-7, 1991) summary of this work which is critical to many aspects of the current work in this project in as follows. The cDNA of human poly(ADP-ribose) polymerase (PADPRP), encoding the entire protein was subcloned into the *Escherichia coli* expression plasmid pYUb. In this expression system, the carboxyl terminus of ubiquitin is fused to the amino terminus of a target protein, in this case PADPRP, stabilizing the accumulation of the cloned gene product. Following induction of the transformed cells, the sonicated extract contained a unique protein immunoreactive with both PADPRP and ubiquitin antibodies and corresponding to the predicted

mobility of the fusion protein in SDS-PAGE. Fusion of ubiquitin to PADPRP increased the yield of PADPRP approximately 10-fold compared to the unfused enzyme. The resulting recombinant fusion protein had catalytic properties which were nearly identical to native PADPRP obtained from mammalian tissues. These properties included specific activity, K_m for NAD, response to DNA strand breaks, response to Mg^{++} , inhibition by 3-aminobenzamide and activity in activity gel analysis. An initial analysis by deletion mutagenesis of PADPRP's functional domains revealed that deletions in the NAD binding domain eliminated all activity, however, partial polymerase activity resulted from deletion in the DNA binding or automodification domains. These activities were not enhanced by breaks in DNA. We further described in this manuscript a colony filter screening procedure designed to identify functional polymerase molecules which will facilitate structure/function studies of the polymerase in the future AFOSR granting period.

In the most recent work in this area, two different PADPRP analogs were prepared to initiate further studies on the structural as well as the enzymatic activity of these abundant nuclear proteins in cells. The expression plasmid pSVNO-3' (i.e. PADPRP lacking 3' untranslated olego nucleotides) was designed in an attempt to overexpress the PADPRP levels in cells since earlier data had indicated that sequences in this region of the PADPRP transcript contributed to the stability and destabilization of this mRNA in cells. To determine the expression of the truncated human transcript in the transfected cell lines, Northern analysis of clones positive for intact integration (clones BP and P6) was performed. Cells were maintained either at semi-confluence (approximately 70% confluence) or logarithmic stage and total RNA was extracted. Duplicate RNA blots were prepared and hybridized with either human or mouse PADPRP cDNA. The pSVNO3' mRNA was only weakly detectable in proliferating cells and undetectable in semi-confluent cells (Fig. 10A, lanes 3, 4). Moreover, the endogenous murine PADPRP mRNA level was decreased in the pSVNO3'-expressing cell lines as compared with the mock-transfected cells.

The PADPRP activity was measured in three cell lines stably transfected with pSVNO3' and two control cell lines. The data indicated that PADPRP activity levels were not substantially altered in the pSVNO3' transfected cells (clones 3'-17, 3'-19, and P6) compared to the two control cell lines. Also, no apparent change in the growth rate of the pSVNO3' transfected cell lines was observed.

A bacterial expression vector containing a human cDNA with a deletion of the 132 bp *Cla* I/*Cla* I region encompassing the NAD-binding site characterized earlier (Cherney, et al), designated pV3, had been altered during this year to construct a eukaryotic expression plasmid. This expression plasmid in eukaryotic cells should cause the expression of a analog PADPRP molecule which has no catalytic activity yet will bind to DNA strand breaks and, perhaps mediate cytotoxicity caused by environmental pesticides, etc. To determine whether the *in vivo* expressed PADPRP mutant mRNA was translated, two Δ Cla-PADPRP cell lines were labeled with [³⁵S]-methionine for 4 hours and immunoprecipitated with antibody to human PADPRP enzyme. A band at the expected molecular weight at 108 kDa, corresponding to Δ Cla-PADPRP truncated by 44 amino acids, was identified below the 113 kDa endogenous murine PADPRP in clones 42 and 49 but not in the control mock-transfected cell lines.

Since the previous data had demonstrated the expression in transfected cells of two forms of PADPRP, one of which presumably an active site mutant, it was interest, to access the influence of these conditions on the overall poly(ADP-ribosylation) levels in the context of the whole cell.

Since the Δ Cla-PADPRP mutant contained an active DNA-binding domain, we anticipated that cell lines, constitutively expressing Δ Cla-PADPRP, might have lowered poly(ADP-ribosylation) capability. Accordingly, the PADPRP activity in several transfected clones, confirmed to have intact integration of cDNA, as well as expression of both mutant PADPRP mRNA and protein was measured by a sonication assay. Contrary to expectations, a 2-fold increase, rather than decrease in polymerase activity, compared with mock-transfected cells and pcD- Δ Cla transfected cells negative for both intact integration and expression of pcD- Δ Cla, was consistently observed.

The second stably transfected cell line characterized during the past grant period, contained a modified PADPRP, which possessed a deletion in the NAD-binding domain. As expected upon

expression of this mutant in *E. coli* we observed that this form of PADPRP was catalytically inactive. As the DNA-binding domain was intact, it was of considerable interest that an apparent increase in polymerase activity was observed in cells expressing the inactive pcD-ΔCla PADPRP mutant. This may prove of considerable experimental usefulness in the future years of the AFOSR project. The apparent enhancement of PADPRP activity by the active site mutant was also confirmed *in vitro*, with partially purified normal polymerase and mutant PADPRP. One plausible explanation for the increased polymerase activity in the transfected cells may involve the accessibility of the automodification domain on the pcD-ΔCla mutant as acceptor sites by the endogenous PADPRP which will lead to exploration of the consequences of inactive polymerase on DNA repair or chromatin structure in future years of the project. Another possibility is that the increased activity observed was a result of stimulation of the active enzyme by protein-protein interaction. Since the pcD-ΔCla NAD-binding domain mutant also has an intact DNA-binding domain, the mutant cell line developed during the past year of the project could potentially compete for DNA strand breaks.

A third avenue, originally suggested to be addressed in Aim I of the application (1992) involved the swapping of the DNA binding zinc finger region of PADPRP with the glucocorticoid receptors zinc finger. This would eventually give the project a potential to also perform a similar swap with cytochrome P452 receptor. This system is involved in activation of toxic compounds of interest to AFOSR. Accordingly, in order to examine the biochemical properties of structural-functional relationships of PADPRP, and to determine the importance of DNA binding for the functions of PADPRP, we have replaced the normal, non-sequence-specific PADPRP zinc finger region with the DNA binding domain from the glucocorticoid receptor (GR) zinc finger region, and expressed this chimeric protein, as well as the native PADPRP fused downstream to ubiquitin in *E. coli* AR58 cells. These two different proteins were then analyzed by Western analysis, gel shift assays, and polymerase assays. The chimeric protein, designated Ub-GR-PADPRP, was recognized by antibodies specific for both PADPRP and the DNA-binding domain of GR, while Ub-PADPRP was only recognized by the PADPRP antibodies. Gel retention experiments performed during the last year have indicated that GR-PADPRP retained the sequence-specific DNA binding characteristics of the native GR; that is, the chimeric protein, but no PADPRP, formed a stable complex with the glucocorticoid DNA response element (GRE). Furthermore, in these preliminary experiments during this period of the grant, this complex was specifically competed by excess GRE oligonucleotide, and destabilized with antibodies to either PADPRP or GR. Finally, both the native PADPRP and the fusion protein GR-PADPRP retain polymerase activity *in vitro* which is inhibited by 3-aminobenzamide (3-AB). However, while the native enzyme is induced by sonicated DNA, the fusion protein is constitutively active, and is not further stimulated by the addition of DNA. These results, obtained during the initial period of the grant, suggest that both the DNA binding domain of the GR and NAD-binding and polymerizing domain of PADPRP retain their native conformation and activity in the chimeric molecule. Further, unlike the native PADPRP DNA binding domain, the corresponding region of the GR lacks the ability to stimulate polymerase activity, suggesting that this induction is dependent upon the specific interaction between the two domains of the native PADPRP. This system characterized during year one of the grant will enable further studies proposed in Aim I to retarget PADPRP to non-break regions of DNA to determine the relationship between poly ADP-ribosylation and cellular functions such as DNA repair and expression of specific genes which may be induced by exposure of cells to cytotoxic agents.

Progress on AIM II; CYTOTOXICITY AND DNA REPAIR STUDIES IN STABLY TRANSFECTED CELL S WITH ALTERED POTENTIAL FOR POLY ADP-RIBOSYLATION - EFFECTS OF ENVIRONMENTAL TOXIC AGENTS

In work published in 1992 during the current granting period (Ding, R. et al., Depletion of Poly(ADP-ribose) Polymerase by Antisense RNA Expression Results in a Delay in DNA Strand Break Rejoining. 1992, *J. Biological Chemistry* 267, pp. 12804-12) we have characterized HeLa cells which are stably transfected with antisense to PADPRP. This work is also supported, in part, by other funds in my program. The depletion of endogenous PADPRP as mediated by induced antisense RNA expression was established by: (i) a progressive synthesis of antisense transcripts in cells as assessed by Northern analysis; (ii) an 80% decrease in activity of the enzyme; and (iii) a greater than 90% reduction in the

cellular content of PADPRP protein, as demonstrated by both immunoblotting and immunohistochemical analysis in intact cells. Several biological parameters were monitored in cells depleted of PADPRP. The chromatin of PADPRP-depleted cells was shown to have an altered structure as assessed by deoxyribonuclease I susceptibility. Cell morphology was also altered. The cells were incubated with the alkylating agent methyl methanesulfonate (MMS), which is analogous in action on DNA to several environmental toxins to be tested in later years of the grant. We utilized alkaline elution method to study DNA repair in these studies. Control cells treated with or without dexamethasone as well as antisense cells without dexamethasone showed essentially the same alkaline elution curves either during short time repair (10 - 45 min) or long repair (5 h) indicating the absence of an indirect effect of dexamethasone, *per se*, on SSB repair. In contrast, the induced PADPRP-as[7] cells showed a significant reduction in the SSB repair rate.

Taken together, our results suggest that PADPRP makes an important contribution to early stages of DNA strand break resolution. Our data also suggest that the PADPRP concentration in HeLa cells is not limiting for DNA repair. This system thus provides a means for analyzing the influence of PADPRP on the reorganization of chromatin structure that occurs subsequent to DNA damage or perhaps during DNA replication and such experiments will be underway during the second and third of the granting period.

C. Technical Journal Publications

Publications Published

Lyn, D., Cherney, B., Lalande, M., Berenson, J., Lupold, S., Bhatia, K., and Smulson, M. A Duplicated Region is Responsible for the Poly(ADP-Ribose) Polymerase Polymorphism on Chromosome 13 Associated with a Predisposition to Cancer. *Am. J. Hum. Genetics* 52, 124-134 (1993).

Ding, R., Pommier, Y., Kang, V., and Smulson, M.E. Depletion of Poly(ADP-Ribose) Polymerase by Antisense RNA Expression Results in a Delay in DNA Strand Break Rejoining. *J. Biol. Chem.*, 267, 12804-12812 (1992).

Blafo, J., Michael, N., Kang, V., Aboul-Ela, N., Smulson, M., Jacobson, M., and Roizman, B. Differences in the Poly(ADP-ribosylation Patterns of ICP4, the Herpes Simplex Virus Major Regulatory Protein, In Infected Cells and In Isolated Nuclei. *J. Virol.* 66, 6398-6407 (1992).

Cherney, B. W., Chaudhry, B., Bhatia, K., Butt, T. R., and Smulson, M. Expression and Mutagenesis of Human Poly(ADP-Ribose) Polymerase as a Ubiquitin Fusion Protein from *Escherichia coli*. *Biochemistry*, 30, 10420-10427 (1991).

Rosenthal, D., Cherney, B., Danielsen, M., and Smulson, M. Expression and Characterization of a Fusion Protein Containing the DNA Binding Domain of the Glucocorticoid Receptor and Catalytic Domain of Poly (ADP-ribose) Polymerase. (in preparation) (1993).

Publications in Preparation

Kang, V., Ntambi, J., Rosenthal, D. and Smulson, M. Expression of Antisense to Poly(ADP-ribose) Polymerase mRNA Inhibits Increases in Enzyme Expression and DNA Replication during Differentiation of 3T3-L1 Preadipocytes. (in preparation) 1993.

Kang, V., Cherney, B., Bhatia, K. and Smulson, M. Construction of Poly(ADP-ribose) Polymerase Mutants and Characterization of Mammalian Cells Stably Transfected with Various Polymerase Mutants. (in preparation) 1993.

Ding, R., Stevens, T., Bohr, V. and Smulson, M. Depletion of Nuclear Poly(ADP-ribose) Polymerase by Antisense Expression: Influences on Genomic Stability and Chromatin Organization. (in preparation) 1993.

D. Professional Personnel

No changes to the professional staff since award of the grant.

E. Interactions

1. Papers Presented at Meetings, Conferences, Seminars

Pero, R., Berglund, G., Christie, N.T., Cosma, G.N., Frenkel, K., Garte, S.J., Jacobson, E.L., Jacobson, M.K., Janzon, L., Seidegård, J., Smulson, M.E., and Troll, W. The Malmö Diet and Cancer Study: The Biomarker Program. Submitted for publication in Journal of Internal Medicine. 1992.

Cherney, B., Bhatia, K., Chaudhry, B., Butt, T., Danielsen, M., and Smulson, M. Expression and Characterization of PADPRP and a Novel Glucocorticoid-PADPRP "Finger Swapped" Protein from *Escherichia coli*., in ADP-Ribosylation Reactions. Edited by Poirier and Moreau, Springer-Verlag, New York. Pages 77-80. 1992.

Kang, V., Haque, S.J., Cherney, B., and Smulson, M. Strategies for Expressing Analogs of PADPRP in Eukaryotic Cells, in ADP-Ribosylation Reactions. Edited by Poirier and Moreau, Springer-Verlag, New York. Pages 73-76. 1992.

Smulson, M.E. Strategies for Studying the Functions of PADPRP Genes on Human Chromosomes 1 and 13, in ADP-Ribosylation Reactions. Edited by Poirier and Moreau, Springer-Verlag, New York. Pages 30-37. 1992.

Smulson, M., Ding, R., Kang, V., Dritschilo, A., Pommier, Y. and Ntambi, J. The Expression of Antisense Transcripts to Poly(ADP-Ribose) Polymerase mRNA Reduces DNA Repair and Modulates Differentiation in 3T3-L1 Preadipocytes. Oji International Seminar on ADP-ribosylation Reaction: Mechanism and Biological Function. Sept. 23-26, 1992, Tsukuba, Japan. Abstract #9.

2. Consultive Functions to Other Laboratories and Agencies

Smulson, M., Ding, R., Kang, V., Dritschilo, A., Pommier, Y., Bhatia, K., Cherney, B. Antisense mRNA to Poly(ADP-Ribose) Polymerase and its Potential Modulation of HD Toxicity. Proceedings of the 1991 Medical Defense Bioscience Review. DTIC Accession # AD B158 588. August, 1991. Pages 17-23.

Ding, R., Stevensner, T., Bohr, V. and Smulson, M. Preferential Gene Repair and HN2 Damage: Depletion of PADPRP by Antisense RNA Expression. Proceedings of the 1993 Medical Defense Bioscience Review. (in preparation) May 10-13, 1993.

Rosenthal, D., Smith, A. and Smulson, M. The Role of PADPRP in the Response of Human Epidermal Cells to Nitrogen Mustard DNA Damage. Proceedings of the 1993 Medical Defense Bioscience Review. (in preparation) May 10-13, 1993.

Smulson, M., Ding, R., Kang, V., Dritschilo, A., Pommier, Y. Ntambi, J. and Rosenthal, D. The Expression of Antisense Transcripts to PADPRP and HD-Induced Preferential DNA Repair and Differentiation. Proceedings of the 1993 Medical Defense Bioscience Review. (in preparation) May 10-13, 1993.

Smulson, M.E. Molecular Biology Basis for the Response of Poly (ADP-rib) Polymerase and NAD Metabolism to DNA Damage Caused by Mustard Alkylating Agents. Contract No.: DAMD17-90-C-0053. Duration: 4/30/90-9/29/94.

Army Proposal Abstract

The enzyme poly(ADP-Ribose) polymerase utilizes NAD as a substrate to modify a variety of nuclear proteins. Its activity is dependent upon DNA with strand breaks. Accordingly, cells exposed to CW agents such as mustards, possess significant depletion of NAD, presumably via accumulation of DNA strand breaks and activation of poly(ADP-ribosylation). It has been hypothesized that the above depletion of NAD in skin cells is responsible for development of subepidermal blisters in personnel exposed to CW agents. During the past two and a half years our project has developed techniques to directly test the latter hypothesis. Accordingly, in this renewal request we wish to continue and complete experiments aimed at genetically altering a model skin tissue to study the effects of mustards on skin cells by the use of molecular biology techniques. We propose to use retroviral-mediated gene transfer to introduce a recombinant human cDNA to sense, antisense orientations, as well as site-directed mutation regions of the poly(ADP-ribose) polymerase gene into cultured hu-

man keratinocytes. This will ultimately allow, in an animal model, the ability to test the hypothesis that this enzyme is pivotal for survival potential for skin cells exposed to CW agents.

The renewal program fits into three different aims. By intent, each aim utilizes recombinant DNA as well as immunological methodology and biochemical analyses as a means to better understand the contribution of Poly(ADP-ribosylation) in skin cells and tissues in response to mustards. These should provide new data bases for assessing the toxic effects of sub-lethal doses of CW agents.

In Aim I, we propose to continue experiments already initiated during the last two years on the construction of retroviral delivery vehicles specifically for keratinocytes in order to allow hyperexpression and intranuclear modulation of poly(ADP-ribose) polymerase activity in keratinocytes-in order to directly test the above hypothesis concerning the toxic effects of NAD lowering in these cells after exposure to mustards. Aim II will utilize these retroviral vectors to engineer keratinocytes to express either antisense sequences and/or site-directed domains of this nuclear enzyme in order to attempt to modulate its activity *in vivo*, in keratinocytes exposed to mustards. In Aim III we will test, first *in vitro* in cultured keratinocytes, and later *in vivo* in nude mice with altered, engineered skin tissue, the influence of ADP-ribosylation on mustard exposure with respect to cell survival and DNA repair. A long term goal will be explored during the renewal period in Aim IV by the potential to utilize transgenic expression system in animals utilizing keratinocyte promoters to modulate poly(ADP-ribosylation) *in vivo* as a stable integration in the genome of intact animals.

We also plan to explore the "similar" use of other DNA repair genes, as they become available for approaches such as above.

F. New Discoveries, Inventions, Patents

Not applicable.

G. Additional Insight

PADPRP requires DNA for activity; a major clue toward ascertaining the function of the enzyme has been the observation described by Benjamin and Gill that the *catalytic activity of PADPRP is directly coordinated to the numbers of strand breaks in DNA*. Thus, relevant to the objectives of AFOSR is the fact that PADPRP causes the poly ADP-ribosylation of nucleosomal proteins in response to a variety of DNA-damaging agents including environmentally toxic agents such as alkylating compounds, aromatic hydrocarbons, UV light and ionizing irradiations.

PADPRP with a homologous DNA binding domain of the glucocorticoid receptor and that this substituted PADPRP molecule is partially catalytically active in the absence of DNA strand breaks. We propose to utilize these substituted PADPRP molecules in two ways: (1). An active site mutant should compete with the endogenous enzyme and allow a better understanding of how this enzyme functions in DNA repair. Also, the substituted PADPRP may associate with specific subsets of genes, namely those which have glucocorticoid responsive sequence elements. This may allow the study of DNA repair of specific genes, as well as providing a route to poly ADP-ribosylate proteins which bind to the regulatory regions of glucocorticoid responsive genes. (2). TCDD, dioxin, a potent environmental contaminant, like glucocorticoid is mediated by an aromatic hydrocarbon receptor which also binds to specific DNA sequences (i.e. dioxin-responsive elements) adjacent to TCDD-responsive genes. There is analogy in the nucleotide and DNA binding domains of the newly characterized dioxin receptors with the glucocorticoid receptors which we have already successfully substituted into PADPRP. Accordingly, one may be able to eventually also substitute the dioxin DNA binding receptor region. This long term goal may allow a better understanding of the proteins that associate with DNA sequences where dioxin binds in cells, and is involved in environmental toxicity.